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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
A complex network of signals between the stroma, the extracellular matrix and the epithelium, and by hormones acting systemically, drive the mammary gland development and function. The tissue organization field theory (TOFT) proposes that alterations of the reciprocal interactions between stroma and epithelium initiate the process of neoplastic transformation of epithelial cells. Our goal is to assess whether the primary target of the carcinogen N-nitroso-methylurea (NMU) is the epithelium, the stroma or both through a protocol of tissue recombination by transplanting mammary gland epithelial cells (MGEC) into mammary gland fat pads (MGFP) previously cleared of epithelium. The animals were divided into 6 groups: (1) NMU-exposed stroma and vehicle (VEH)-exposed MGEC; (2) NMU-exposed stroma and NMU-exposed MGEC; (3) VEH-exposed stroma and NMU-exposed MGEC; (4) VEH-exposed stroma and VEH-exposed MGEC; (5) positive control (intact virgin rat exposed to NMU); (6) negative control (exposed to VEH). Results: the tumor incidence was G1 83.3%, G2 85.7%, G3, 4 and 6 0%, G5 100%. Our results show that the stroma, rather than the epithelial cells, may be responsible for the development of a neoplasia. This novel concept in carcinogenesis will provide clues to be applied to more rational study of breast cancer.

14. SUBJECT TERMS
stroma-epithelium interaction, mammary gland carcinogenesis, breast cancer

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Introduction

For almost a century now, the view that carcinogenesis takes place at the cellular and subcellular levels has been the prevalent one. The implicit premises of this hypothesis, called the Somatic Mutation Theory are: 1) cancer originates at the single cell level; 2) tumor initiation involves the stable mutations of DNA by carcinogens (1), and 3) mutations must result in an increase of the proliferative rate of the neoplastic cell (2).

Although the proponents of the ***somatic mutation theory*** of carcinogenesis have readily acknowledged that, in some instances, epigenetic mechanisms may be sufficient to explain carcinogenesis, the study of tumor initiation has been focused at the genome level.

Alternatively, epigenetic mechanisms similar to those occurring during histogenesis and organogenesis have been proposed to be at the core of carcinogenesis (3). During embryogenesis, adjacent stroma and epithelia exert instructive influences on each other resulting in organ formation. These units are called the "morphogenetic fields". It has been postulated that these units of tissue maintenance and/or organization are three-dimensional and carry positional and historical information. Interactions between epithelium and stroma initiate a flow of information that acts to regulate many fundamental processes throughout development. These include cell migration, morphogenesis, and modulation of growth and differentiation programs of many specialized cell types (4). The contribution of stroma to early events in carcinogenesis has recently begun to be appreciated. It has been postulated that cancer is a physiological response to an abnormal environment (5).

In addition, Bissell et al. have stated that the unit of function in higher organisms is neither the genome nor the cell alone but the complex, three-dimensional tissue. This is because there are bi-directional connections between the components of the cellular microenvironment and the nucleus. These connections are made via membrane-bound receptors and transmitted to the nucleus, where the signals result in modifications to the nuclear matrix and chromatin structure and lead to selective gene expression. Thus, cells need to be studied "in context", within the proper tissue structure, if one is to understand the bi-directional pathways that connect the cellular microenvironment and the genome (6).

There are several lines of evidence showing that carcinogenesis may be mediated by alterations of tissue organization. In the mammary gland, stroma-epithelial reciprocal influences have been shown to be essential for proper development of the gland during the embryonic and postnatal stages. In "spontaneous" and agent-mediated carcinogenesis there is a disruption of the normal interactions that take place among cells in the parenchyma and subjacent

stroma of an organ. This disturbance results in functional and structural changes in the affected tissue/organ.

The ***tissue organization field theory*** of carcinogenesis and neoplasia states that carcinogens disrupt the flow of information between the stroma and the parenchyma and/or among cells within those tissues. The temporary or permanent effects of carcinogens on the intracellular structures and components while variably deleterious to each of them are not directly responsible for the development of a neoplasia (3).

Specialized microenvironments composed of insoluble extracellular matrix and soluble factors, mediate epithelia-stromal interactions and play a pivotal role in normal tissue development and function (7). In the terminology of developmental biology, the microenvironment generated by the abnormal epithelial-stromal interactions may be considered "permissive" for the emergence of hyperplasia, displasia, and neoplasia (3;5). Moreover, the neoplastic behavior of cells can be reversed when they are placed in normal environments (8).

My research proposal aims at developing an *in vivo* model to study mammary gland carcinogenesis at a tissue level of organization. The goal is to test the three competing hypotheses, namely, 1) that the primary target of the chemical carcinogen nitrosomethylurea (NMU) is the stroma, 2) that the primary target is the epithelium, and 3) that both the epithelium and the stroma need to be exposed to the carcinogen.

The proposal aim was to assess whether the primary target of NMU in NMU-induced mammary carcinogenesis is the epithelium, the stroma, or both through establishing a protocol of tissue recombination by transplanting epithelial cells into mammary gland fat pads that have been previously cleared of epithelium. Recombinants will be produced between 1) vehicle exposed stroma and vehicle-exposed epithelium, 2) vehicle-exposed stroma and NMU-exposed epithelium, 3) NMU-exposed stroma and vehicle-exposed epithelium, and 4) NMU-exposed stroma and NMU-exposed epithelium. The number of mammary carcinomas arising from these recombinants and from intact animals treated with NMU (positive control) and with vehicle (negative control) will be compared.

Body

Experimental design: Virgin 55 day-old Wistar-Furth rats were used as epithelial cell donors.

The experimental groups are shown in Table 1. Both NMU (50mg/100g body weight in 0.85% NaCl pH 5) and vehicle (0.85% NaCl pH 5) injections were done intraperitoneally. The epithelial cell transplantation was performed 1 week after the NMU or vehicle injection. Fifty thousand cells/10μl were injected into each cleared fat pad. The animals were palpated once a week, starting one month after the cell injection.

Table 1: Experimental design for stroma-epithelium recombination. The animals are sacrificed when the tumors reach 1-1.5 cm or 9 months after cell transplant whichever comes first.

	Cleared fat pad at 21 days of age	**NMU exposure at 52 days of age**	**Epithelial cell transplantation**
Group 1	Yes	Yes (50mg/100g bw *)	Yes (vehicle- exposed cells)
Group 2	Yes	Yes	Yes (NMU-exposed cells)
Group 3	Yes	No	Yes (NMU-exposed cells)
Group 4	Yes	No	Yes (vehicle-exposed cells)
Group 5	No	Yes	No
Group 6	No	No	No

* bw: body weight

Clearing of the mammary fat pad: The surgery was performed following the procedure previously reported by DeOme et al (9). Using the nipple as a guide, a small portion of the fat pad containing the epithelial tissue was removed and fixed for a whole mount preparation as a quick way to assess the presence of the epithelium (Figure 1). The survival rate of the animals after the surgery was 100%.

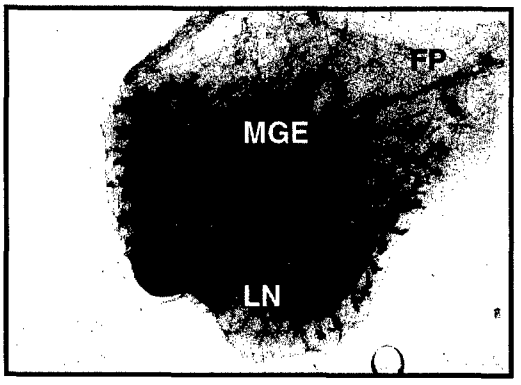

Figure 1: Rat mammary gland at 21 days of age. Whole mount preparation. Magnification: 2x

LN: Lymph node
FP: fat pad
MGE: mammary gland epithelium

In order to check whether or not the fat pad was completely cleared, the animals were allowed to reach puberty and, in a post-puberal stage, a new whole mount preparation was done with the remaining fat pad (Figure 2).

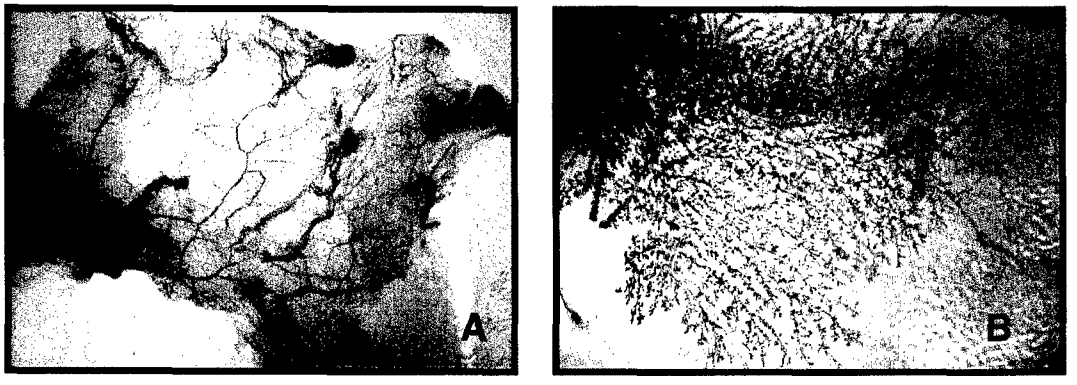

Figure 2: A) Fat pad removed 2 months after being cleared of epithelial cells. B) Age-matched intact fat pad containing a full-developed mammary gland. Whole mount preparation. Magnification: 0.6x

***Isolating and propagating mammary epithelial cells*:** The mammary epithelial cells are isolated using a method adapted from Hahm and Ip (10). The protocol includes tissue dissociation using collagenase and pronase and filtering through a Nitex cloth. The epithelial cells are grown in serum-free, phenol red-free DMEM/F12 medium supplemented with insulin, progesterone, epidermal growth factor, prolactin, fatty acid free bovine serum albumin, hydrocortisone, transferin, ascorbic acid, and gentamicin. The cells were seeded in matrigel-coated 6-well

plates. Stromal cell grow very poorly in serum-free medium; moreover, these cells are easily detached using a trypsin/EDTA solution. Thus, after 4 weeks in culture the number of fibroblasts is very low. We have tested the purity of the epithelial cells preparations using an anti-cytokeratin antibody to recognize epithelial cells and an anti-vimentin to recognize stromal cells. We have confirmed the epithelial origin of the growing colonies and the percentage of contamination with stromal cells is less than 20%.

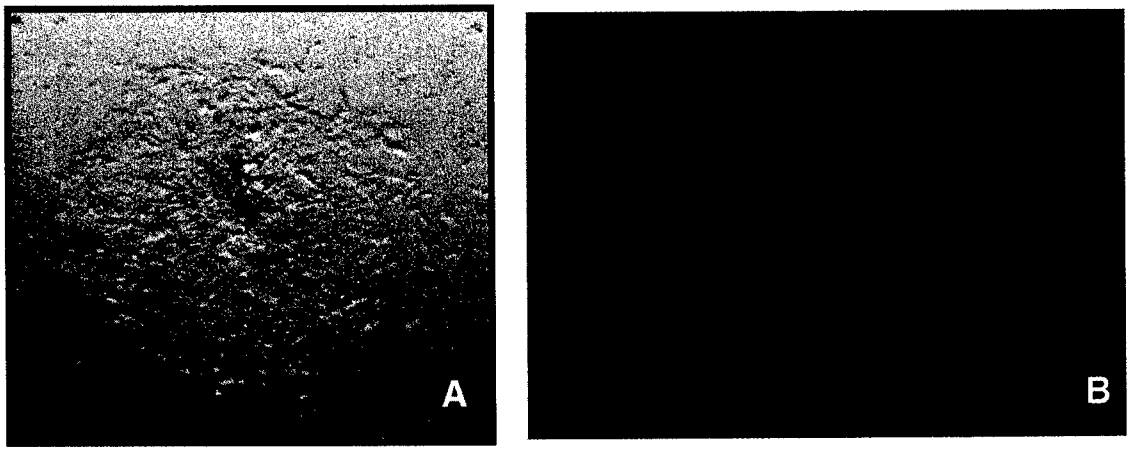

Figure 3: Mammary epithelial cells in culture. **A)** Primary culture of mammary epithelial cells. **B)** Cell characterization using an anti-cytokeratin antibody, a specific marker for epithelial cells. Red fluorescence: cytokeratin; blue fluorescence: DNA-specific dye Hoechst. Magnification: 5x (A); 20x (B)

One week after the NMU or vehicle injection, the animals were transplanted with mammary gland epithelial cells that were either NMU-exposed or vehicle-exposed, following the protocol described by Abrams et al (11). The cells, at a concentration of $5x10^5/10\mu l$ were injected into each cleared fat pad using a 100µl Hamilton syringe. Epithelial cells were exposed *in vitro* either to NMU or vehicle following the protocol described by Miyamoto et al (12).

Tissue processing and immunohistochemistry: The mammary gland whole mount was prepared according to Thompson et al. (13). The tumors were fixed using phosphate buffered 10% formaldehyde and paraffin embedded.

Key Research accomplishments

Tumor latency period: The tumor latency period for the positive control Group 4 was according to the literature. There was no difference between Group 5 and Group 2 at the time 50% of the animals bore tumors. Although the latency period in animals from Group 1 was longer, the detection of palpable lesions was steady. The lesions palpated later do not correspond to spontaneous mammary tumors as these tumors appear in Wistar-Furth rats older than 24 months.

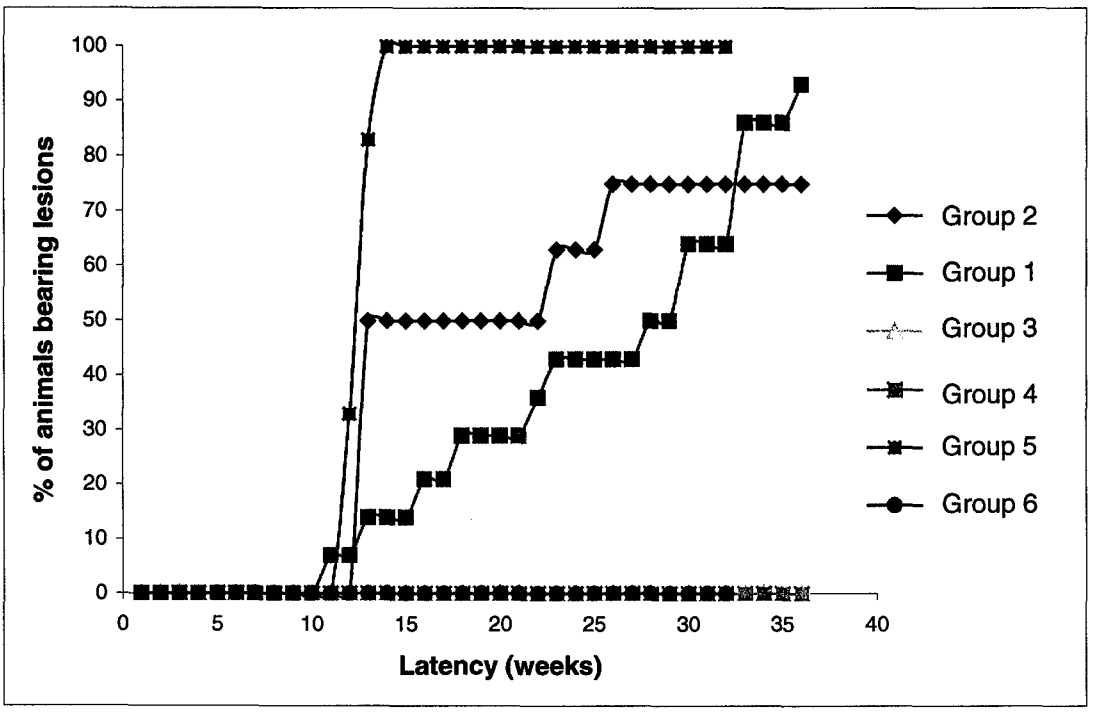

Figure 4: Tumor latency period

Tumor incidence: 83.3% of the animals from Group 1 and 85.7% of Group 2 developed tumors. In animal from Group 3 and Group 4 no tumors were developed. All animals (100%) from the positive control Group 5 developed tumors, whereas none of the negative control Group 6 did (Figure 5).

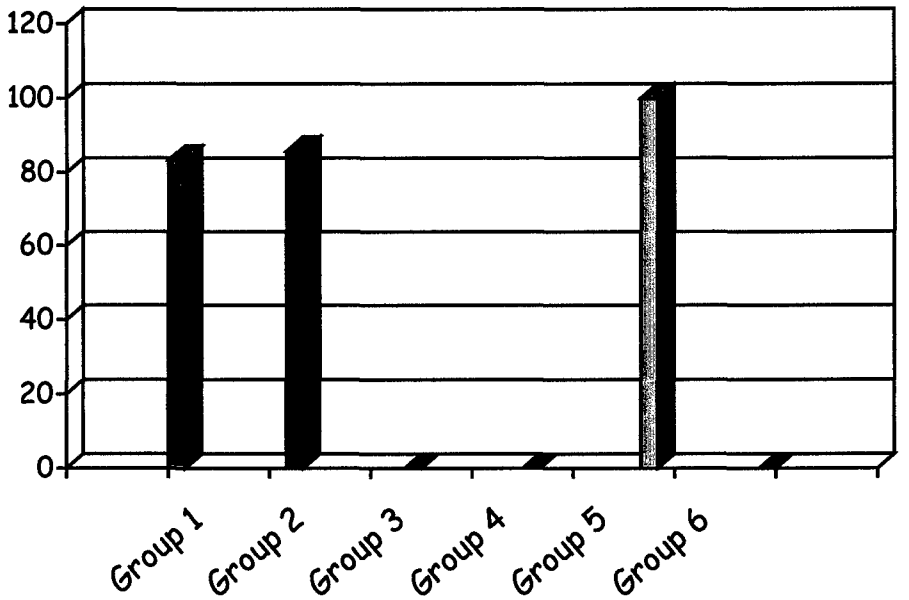

Figure 5: Percentage of animals bearing mammary gland tumors

There was no significant difference between the animals exposed to NMU transplanted with vehicle-treated cells and those in which both, the stroma and the transplanted epithelial cells were exposed to the NMU.

Figure 6 shows mammary gland whole mounts from Group 1 and Group 4 animals. The injected cells were able to form a whole mammary gland and repopulate the entire fat pad.

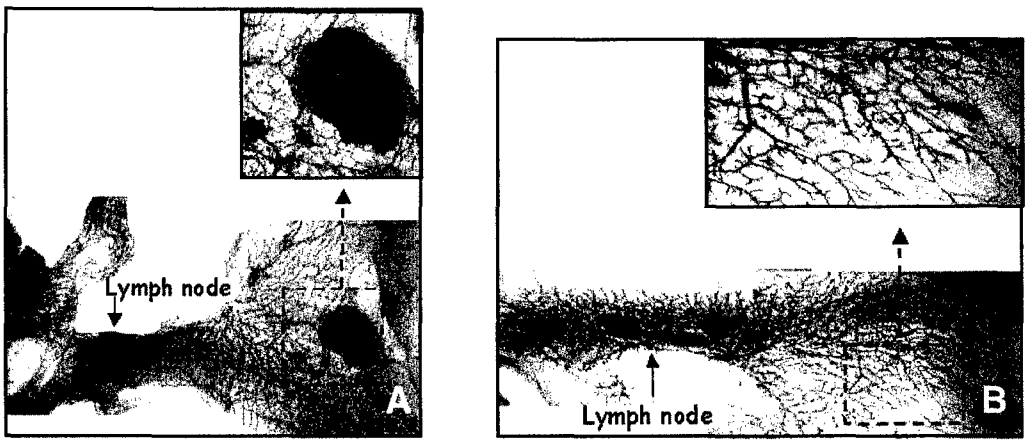

Figure 6: Examples of mammary gland whole mounts from tissue recombinant between NMU-exposed stroma and vehicle-exposed epithelial cells **(A)** and vehicle-exposed stroma and NMU-exposed epithelial cells. Magnification: 0.6x

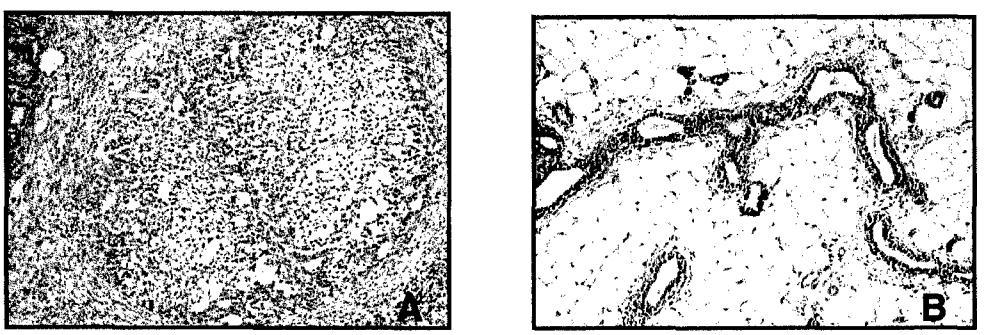

Figure 7: Histological sections representing a tumor **(A)** and a normal mammary gland **(B)**. The tumor represented in **(A)** is a ductal carcinoma *in situ*, cribiform type. Hematoxilin-eosin staining. Magnification: 20x

Tissue evaluation: We are currently assessing the histopathology of the tumors using hematoxylin-eosin stained sections. The branching pattern, the percentage of tissue occupied by ducts, terminal end buds and alveolar structures in the whole mount are being evaluated as well.

Tumor characterization includes histochemical and immunohistochemical staining such as Periodic acid Schiff (PAS) used to evaluate the extracellular matrix and its distribution along the stroma, toluidine blue to recognize mast cells, cytokeratin, vimentin, desmin and BrdU incorporation.

Most of the tumors have been classified as ductal carcinoma *in situ* with a papillary and/or cribiform pattern (Figure 8). Among the main histological changes in the stroma we have observed an increase in the extracellular matrix deposition, replacement of the normal fat pad for fibroblasts and infiltration of leukocytes (eosinophils, plasma cells, mast cells) (Figure 9).

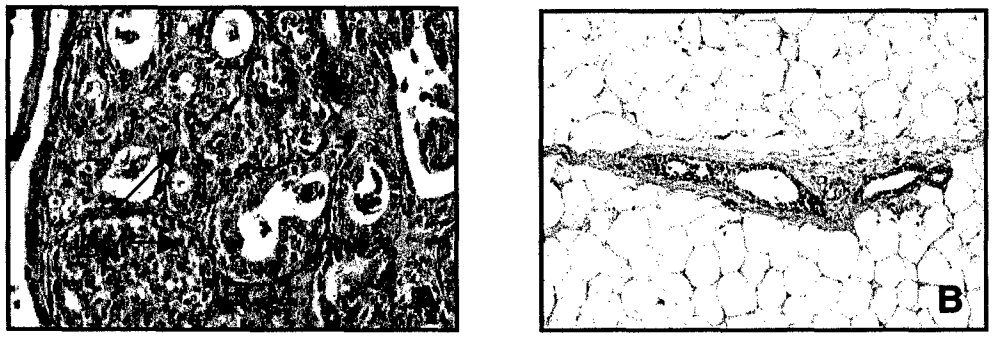

Figure 8: Histological sections of a mammary tumor **(A)** and a normal gland **(B).** Note the irregular and abundant extracellular matrix (ECM) deposits. Thickening of the basement membranes (BM) is also a common finding in tumors. PAS staining. Magnification 20x

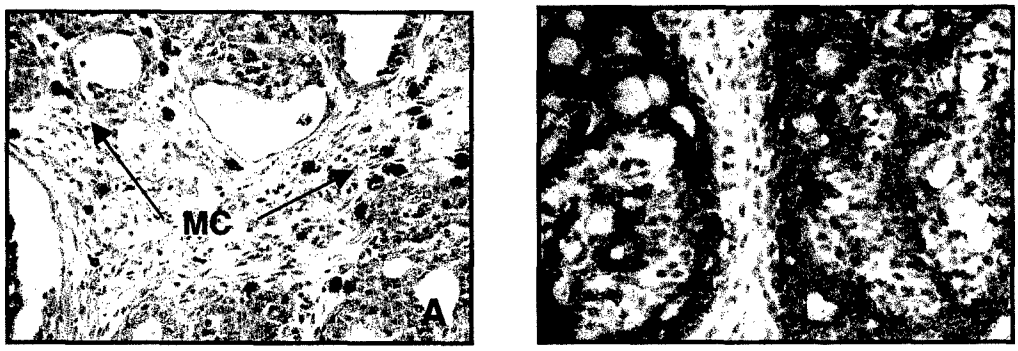

Figure 9: **A)** Infiltration of mast cells (MC) and eosinophils are seen mainly in the stroma. Toluidine Blue stains the mast cells polychromatofilic granules. **B)** Immunodetection of cytokeratin (brown), a specific marker of the epithelial origin of the tumor cells. Counterstaining: Harris' hematoxilin. Magnification 20x

Reportable outcomes

Gordon Research Conference in Mammary gland biology. Poster presentation "Mammary gland stroma contributes to epithelial cell neoplasia". Maricel V. Maffini, Janine M. Calabro, Carise Wieloch, Carlos Sonnenschein, Ana Soto. Il Ciocco (Italy). April 2002

The 12th International Conference of the International Society of Differentiation. "Mammary gland stroma is responsible for epithelial cell neoplasia". Maricel V. Maffini, Janine M. Calabro, Carise Wieloch, Carlos Sonnenschein, Ana Soto. Lyon (France). September 2002

Conclusions

Our results suggest that the stroma, rather than the epithelium, is the target of the carcinogen. Moreover, the *in vitro* exposure of the mammary gland epithelial cells to a chemical carcinogen such as NMU did not induce tumor formation neither increase the tumor incidence when transplanted into an NMU-exposed stroma.

The lack of a significant difference in the tumor incidence between the animals exposed to NMU transplanted with vehicle-exposed epithelial cells and those in which both, the stroma and the epithelial cells were exposed to NMU, suggests that the stroma would be the tissue component responsible for tumor formation. The exposure of isolated epithelial cells to a carcinogen would not be sufficient to give rise to a tumor.

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